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DNA supercoiling changes the spacing requirement of two *lac* operators for DNA loop formation with *lac* repressor

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We have used a gel retardation assay to investigate the influence of DNA supercoiling on loop formation between *lac* repressor and two *lac* operators. A series of 15 DNA minicircles of identical size (452 bp) was constructed carrying two *lac* operators at distances ranging from 153 to 168 bp. Low positive or negative supercoiling ($\sigma = \pm 0.023$) changed the spacing between the two *lac* operators required for the formation of the most stable loops. This reveals the presence of altered double helical repeats (ranging from 10.3 to 10.7 bp) in supercoiled DNA minicircles. At elevated negative supercoiling ($\sigma = -0.046$) extremely stable loops were formed at all operator distances tested, with a slight spacing periodicity remaining. After relaxation of minicircle-repressor complexes with topoisomerase I one superhelical turn was found to be constrained in those minicircles which carry operators at distances corresponding to a non-integral number of helical turns. This indicates that DNA loop formation can define local DNA domains with altered topological properties of the DNA helix.

Key words: *lac* repressor/DNA loop/gel retardation/DNA supercoiling

Introduction

Evidence in favour of DNA looping has been presented in a variety of prokaryotic and eukaryotic systems (for reviews, see Ptashne, 1986; Robertson, 1987; Schleif, 1987). The occurrence of DNA looping was concluded (i) *in vivo* from the cooperative action of distant protein binding sites (Dunn *et al.*, 1984; Martin *et al.*, 1986; Takahashi *et al.*, 1986; Dandanell *et al.*, 1987), and (ii) *in vitro* from DNase I footprinting experiments (Hochschild and Ptashne, 1986) and electron microscopy (Griffith *et al.*, 1986; Theveny *et al.*, 1987). In most of these systems DNA looping depended on the correct spacing between the two protein binding sites: an insertion or a deletion of 5 bp between the two sites impaired cooperativity or prevented loop formation.

By all these criteria DNA looping was demonstrated to exist in the *lac* system. *lac* repressor is a tetrameric protein. Kania and Müller-Hill (1977) showed that two subunits are sufficient for binding and proposed that *lac* repressor can bind simultaneously to two *lac* operators. The destruction of the second *lac* operator decreases repression of the *lac* operon (Eismann *et al.*, 1987), whereas insertion of an additional *lac* operator can increase repression (Besse *et al.*, 1986; Mossing and Record, 1986). Complex formation has been observed between a single repressor and two small DNA fragments containing a *lac* operator (Culard and Maurizot, 1981; O'Gorman *et al.*, 1980). Footprinting experiments revealed cooperative binding of *lac* repressor to two *lac* operators resulting in the looping of the intervening DNA as evidenced by the occurrence of hypersensitive sites (Borowiec *et al.*, 1987; Krämer *et al.*, 1987). DNA loops ranging in size from 74 to 535 bp could be visualized by electron microscopy (Krämer *et al.*, 1987). These results, combined with the well known physiology of the *lac* repressor (Müller-Hill, 1975; Miller, 1978; Lehming *et al.*, 1987), make the *lac* system a paradigm to study the requirements and consequences of DNA looping.

In an initial analysis we characterized DNA loops formed upon binding of *lac* repressor to linear DNA carrying two *lac* operators (Krämer *et al.*, 1987). However, DNA is known to be supercoiled in bacteria and some recent studies pointed to a role of supercoiling in DNA loop formation. In the *ara* system of *Escherichia coli* the upstream site *ara*O_2 requires a supercoiled template to exert its effect on the CRP-dependent induction of the *ara*BAD promoter (Hahn *et al.*, 1986). Supercoiling also facilitates the formation of DNA loops in the *lac* system between the first and the third operator (Borowiec *et al.*, 1987) and supposedly between the first and the second operator (Whitson *et al.*, 1987a,b).

To gain more insight into the role of supercoiling we examined DNA looping by *lac* repressor using the technique of topoisomer gel retardation (Nordheim and Meese, 1988). Topoisomeric DNA minicircles, which differ only in their linking number (Lk), can be separated in the same low percentage acrylamide gels (Shore and Baldwin, 1983b; Horowitz and Wang, 1984; Zivanovic *et al.*, 1986) which are used for gel retardation assays to separate DNA-protein complexes from protein-free DNA. (Fried and Crothers, 1981; Garner and Revzin, 1981.) Topoisomers, which differ in their linking number Lk by $\Delta Lk = 1$, can be separated electrophoretically since the difference in linkage is partitioned into changes in helical twist, ΔTw, and changes in spatial writhe, ΔWr, according to the relation (Fuller, 1971; Crick, 1976):

$$\Delta Lk = \Delta Tw + \Delta Wr$$

We show here that (i) low positive or negative supercoiling changes the spacing requirement for DNA looping, (ii) moderate negative supercoiling increases the stability of DNA loops and (iii) one superhelical turn can be constrained by loop formation.

Results

We constructed a series of 15 DNA minicircles, identical

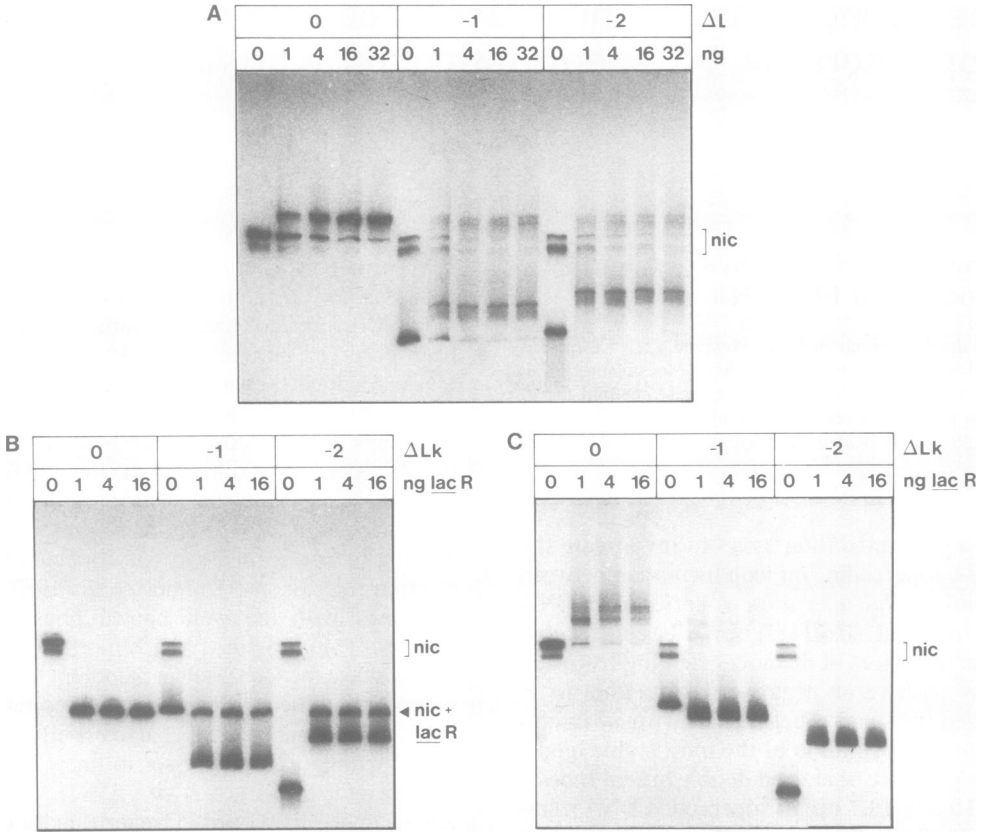

Fig. 1. Titration of *lac* operator-carrying DNA minicircles with *lac* repressor. Purified topoisomers (~0.1 ng) with the indicated linking number difference (ΔLk) carrying: (**A**) one 'ideal' *lac* operator, (**B**) two 'ideal' *lac* operators at a distance of 158 bp and (**C**) two 'ideal' *lac* operators at a distance of 163 bp were incubated with the indicated amounts of *lac* repressor and the mixtures were analyzed by polyacrylamide gel electrophoresis. Positions of the nicked minicircles (nic) are indicated.

in size (452 bp), carrying two 'ideal' *lac* operators at distances varying from 153 to 168 bp. Approximately 200 bp of these minicircles were derived from the *lac* promoter region, with one 'ideal' *lac* operator replacing the wild-type first operator and the second 'ideal' *lac* operator located in a polylinker inserted upstream of the wild-type third *lac* operator (Besse *et al.*, 1986; Krämer *et al.*, 1987). For comparison a 422-bp minicircle was used carrying only one 'ideal' *lac* operator in place of the wild-type first operator. In all experiments described below the individual topoisomers were purified by polyacrylamide gel electrophoresis (Zivanovic *et al.*, 1986) prior to use in topoisomer binding assays (Nordheim and Meese, 1988). Thus in all cases we analysed individual topoisomers, which were unavoidably contaminated by some nicked DNA. The nicked DNA is represented in our gels by two bands (see Figures 1, 3–6), a finding for which we have no explanation.

Binding of lac repressor to DNA minicircles containing one or two lac operators

Figure 1A shows a gel retardation experiment. Relaxed, −1 and −2 topoisomers carrying only one 'ideal' *lac* operator are analysed with increasing amounts of *lac* repressor. An apparent increase in repressor affinity to the increasingly supercoiled topoisomers (0 to −2) can be deduced from the lower amounts of *lac* repressor necessary to shift the DNAs to the retarded positions. In order to further characterize this supercoil-dependent increase in affinity, we determined dissociation rate constants of complexes between *lac* repressor and the three topoisomers, as described in Materials and methods. The half-lives of the complexes increase from 5 min for the relaxed, and 11 min for the −1 topoisomer to 22 min for the −2 topoisomer (data not shown).

To understand the general electrophoretic migration behaviour of minicircles containing DNA loops we initially analysed two sets of minicircles. The first set contained topoisomers (0, −1, −2) carrying the two *lac* operators at a distance of 158 bp. This operator spacing was shown to be optimal for DNA loop formation by *lac* repressor in linear DNA (Krämer *et al.*, 1987). Figure 1B shows the titration of these minicircles with *lac* repressor. Interestingly, the electrophoretic mobilities of the relaxed and the −1 topoisomers were increased by binding of *lac* repressor. We take this as evidence for loop formation assuming that the minicircles are constricted by the simultaneous binding of *lac* repressor to the two *lac* operators. This constriction is interpreted as reducing the hydrodynamic volume of the molecule, thus allowing more rapid migration through the gel matrix. The constriction of relaxed minicircles by the simultaneous binding of *lac* repressor to two *lac* operators can be demonstrated directly by electron microscopy (Figure 2).

The complexes derived from the nicked DNA cannot be distinguished from the complexes with the relaxed topoisomers (see also Figures 3–6), which indicates that the topoisomer denoted as ΔLk = 0 is indeed near to relaxation under these conditions. The faster migrating −2 topoisomer is retarded upon binding of *lac* repressor.

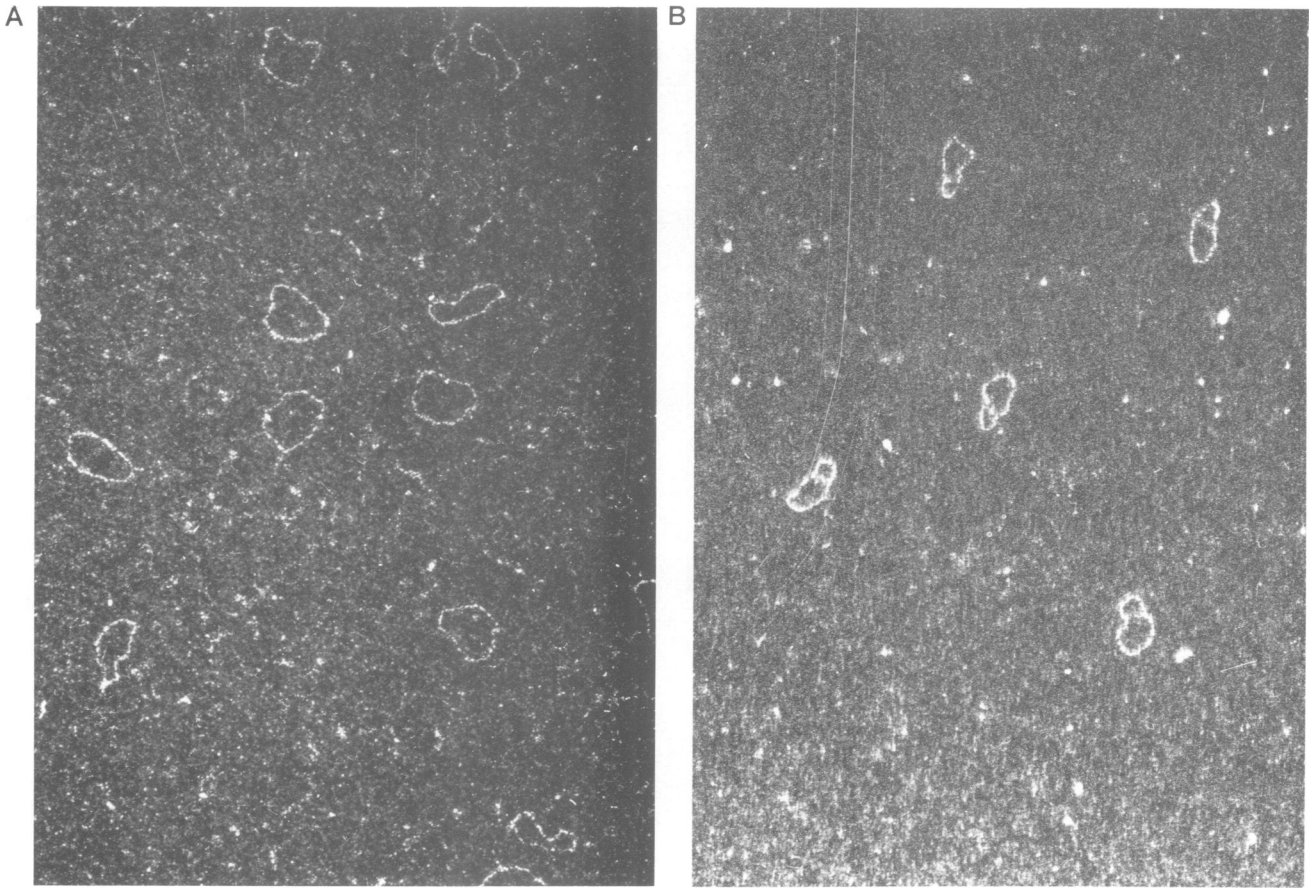

Fig. 2. Electron micrographs of DNA minicircles. Purified relaxed topoisomers carrying two *lac* operators at a distance of 156 bp were incubated in the absence (**A**) or presence (**B**) of *lac* repressor. The final magnification was × 110 400 or × 103 500 respectively.

The second set of minicircles was derived from constructs which carried the two *lac* operators at a distance of 163 bp (Figure 1C). At this distance loops have been shown to be extremely unstable with linear DNA because the two operators are on opposite faces of the DNA helix (Krämer *et al.*, 1987). Titration of the relaxed topoisomer ($\Delta Lk = 0$) shows the appearance of two retarded complexes, which we assume to correspond to single-site binding and tandem formation respectively, as was observed with linear DNA (Krämer *et al.*, 1987). The diffuse smear which can be seen may result from other complexes, like sandwich structures or DNA loops, which are not stable enough to be resolved as a distinct band in the gel. In contrast, binding of *lac* repressor to the -1 topoisomer yields a sharp band which migrates faster than the free DNA, indicating constriction by DNA loop formation. Thus supercoiling allows the formation of stable loops not observed with relaxed DNA when the operators are separated by 163 bp. The -2 topoisomer is equally retarded in a sharp band.

Loop formation between differently spaced operators within DNA minicircles of $\Delta Lk = 0$

To investigate further how supercoiling influences DNA loop formation we used a series of 15 DNA minicircles carrying two *lac* operators at distances increasing from 153 to 168 bp. In Figure 3A the electrophoretic mobilities of the complexes formed between *lac* repressor and the different relaxed topoisomers ($\Delta Lk = 0$) are compared. Sharp bands are seen with the DNA minicircles carrying operators at distances of 156, 158, 159 bp, and 167 and 168 bp, which we interpret to represent complexes with stable DNA loops. This was concluded from their high stability compared to the topoisomers carrying only one 'ideal' *lac* operator (see below) and their increased electrophoretic mobilities. The diffuse bands found with minicircles carrying operators at distances of 154, 161 and 165 bp are believed to result from a rapid equilibrium between single-site binding and DNA loops.

Due to the expected extremely high affinities of the complexes it was not possible to measure their equilibrium binding constants (Whitson *et al.*, 1987a). Instead, we used two methods to compare the stabilities of the different complexes. First, we determined the dissociation of the preformed complexes in the presence of a 10 000-fold molar excess of competitor DNA containing an 'ideal' *lac* operator (Riggs *et al.*, 1970). After 2 h of dissociation the highest amount of complexes between *lac* repressor and the relaxed topoisomers were found retained on constructs carrying the two *lac* operators at 158- and 168-bp distances (Figure 3B). Second, we decreased the affinity of *lac* repressor to *lac* operator ~1000-fold by adding the *lac* inducer IPTG to the incubation mixture (Barkley and Bourgeois, 1978; Daly and Matthews, 1986). To ensure that equilibrium was reached incubation was prolonged for 3 h. The complexes formed between *lac* repressor (200 ng) and the relaxed topoisomers in the presence of 3 mM IPTG are shown in Figure 3C. The same maxima of stability, with respect to operator spacing,

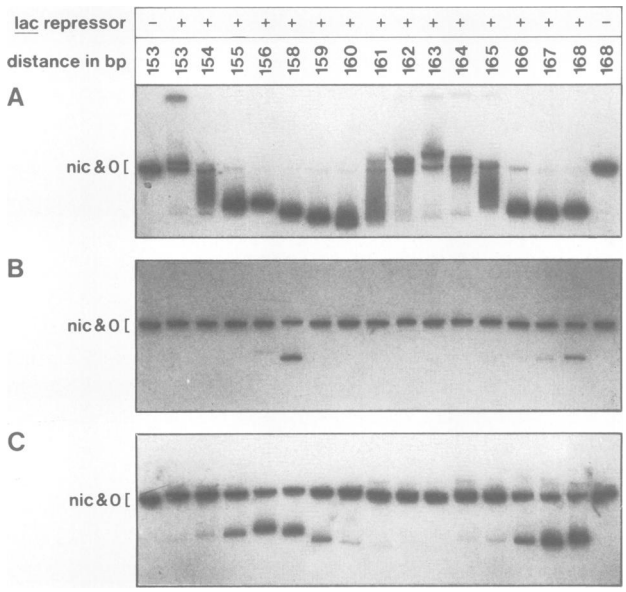

Fig. 3. Influence of the distance between the two *lac* operators on the formation and stability of complexes between *lac* repressor and relaxed topoisomers. Purified relaxed topoisomers (~0.1 ng) with the indicated distances between the two *lac* operators were incubated with 20 ng of *lac* repressor for 15 min. Incubation was continued for 2 h in the absence (**A**) or presence (**B**) of 10 pmol of a *lac* operator carrying competitor DNA. (**C**) The topoisomers were incubated with 200 ng of *lac* repressor in the presence of 3 mM IPTG for 3 h. Incubation, electrophoresis and autoradiography were performed as described in Materials and methods. Positions of free nicked (nic) and relaxed (0) minicircles are indicated.

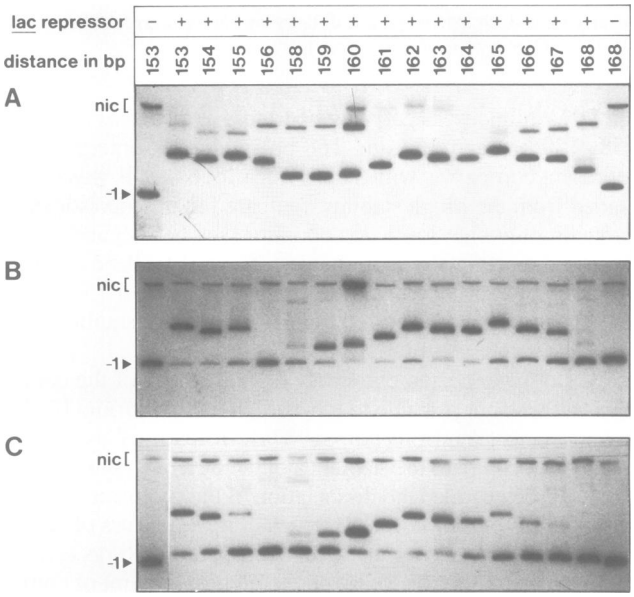

Fig. 4. Influence of the distance between the two *lac* operators on the formation and stability of complexes between *lac* repressor and the −1 topoisomers. Purified −1 topoisomers (~0.1 ng) with the indicated distances between the two *lac* operators were incubated with 20 ng of *lac* repressor for 15 min. Incubation was continued for 3 h in the absence (**A**) or presence (**B**) of 10 pmol of a *lac* operator carrying competitor DNA. (**C**) The topoisomers were incubated with 200 ng of *lac* repressor in the presence of 3 mM IPTG for 3 h. In order to allow separation of all complexes from the −1 topoisomers the gel system was modified by the addition of 5 mM $MgCl_2$ as described Materials and methods. Positions of free nicked minicircles (nic) and free −1 topoisomers (−1) are indicated.

were observed with both methods. Thus with relaxed minicircles stable loop formation occurs at the same operator distances that were found to be optimal in linear DNA fragments (Krämer *et al.*, 1987).

Loop formation between differently spaced operators within DNA minicircles of ΔLk = −1

Figure 1 demonstrates that the complexes formed between *lac* repressor and the −1 topoisomers can migrate faster than the uncomplexed DNA under our standard electrophoresis conditions. Under these conditions the minicircles with operator spacings of 155, 161 and 167 bp form complexes with mobilities very similar to those of the free −1 topoisomers (data not shown). To achieve good separation between bound and free DNA we therefore modified the electrophoresis conditions by the addition of 5 mM $MgCl_2$. Mg^{2+} ions cause a change in twist so that the DNA helix is more tightly wound. Thus, with a fixed linking number, the minicircles exhibit an altered writhe resulting in changed electrophoretic migration (Shure and Vinograd, 1976). In this modified gel system all the complexes formed with the −1 topoisomers yield sharp bands with lower mobility than the free DNA (Figure 4A). When the complexes are challenged by the addition of an excess of unlabelled *lac* operator, new minima (156/157 bp) and maxima (161/162 bp) for stability of the complexes were found with respect to operator spacing (Figure 4B); this contrasts with the maxima of 158 and 168 bp found for the relaxed topoisomers. Additionally the overall stabilities of the complexes were increased over those of complexes with the relaxed topoisomers. The dissociation kinetics of the complexes be-

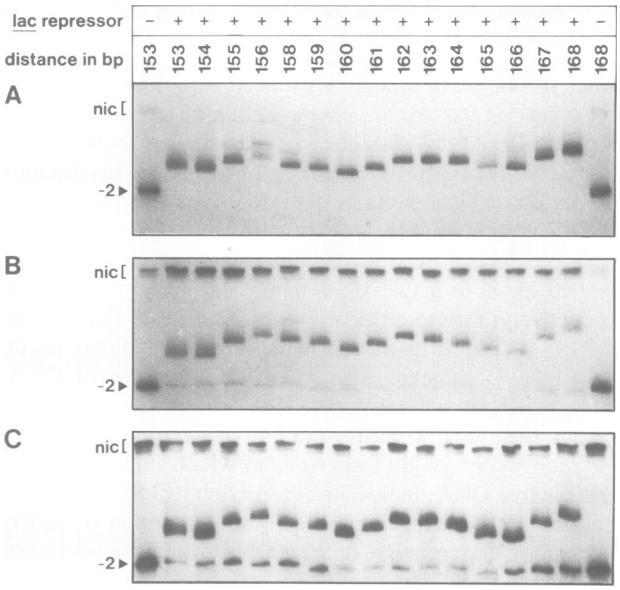

Fig. 5. Influence of the distance between the two *lac* operators on the formation and stability of complexes between *lac* repressor and the −2 topoisomers. Purified −2 topoisomers (~0.1 ng) with the indicated distances between the two *lac* operators were incubated with 20 ng of *lac* repressor for 15 min. (**A**) The mixtures were directly subjected to electrophoresis. (**B**) The incubation was continued for 11 h in the presence of 10 pmol of a *lac* operator carrying competitor DNA. (**C**) The topoisomers were incubated with 40 ng of *lac* repressor in the presence of 3 mM IPTG for 3 h. Incubation, electrophoresis and autoradiography were performed as described in Materials and methods. Positions of free nicked minicircles (nic) and free −2 topoisomer (−2) are indicated.

tween *lac* repressor and the −1 topoisomers were also analysed by applying the incubation mixtures after 1, 2, 3, 4, 5 and 6 h directly to a running polyacrylamide gel (Fried and Crothers, 1981). The percentage of the bound DNA was determined densitometrically from the autoradiographs of the dried gels and these data were used to determine dissociation rates (Riggs *et al.*, 1970). The half-life of the complexes between *lac* repressor and the −1 topoisomers with an operator distance of 161 bp was found to be >20 h compared to a half-life of 2 h for the most stable complexes formed between *lac* repressor and the relaxed topoisomers carrying operators separated by 158 bp (data not shown). The increase in stability and the shift in optimal operator spacing were also found when the complexes were formed in the presence of 3 mM IPTG (FIgure 4C).

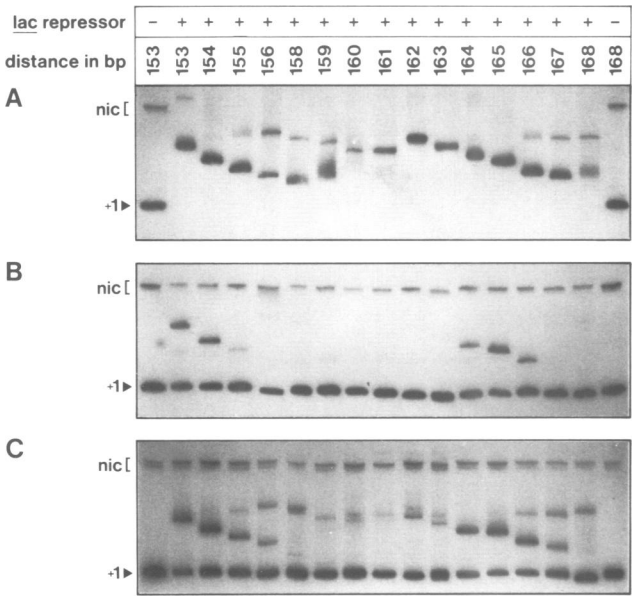

Fig. 6. Influence of the distance between the two *lac* operators on the formation and stability of the complexes between *lac* repressor and +1 topoisomers. Purified +1 topoisomers (~0.1 ng) with indicated distances between the two *lac* operators were incubated with 20 ng of *lac* repressor for 15 min. Incubation was continued for 2 h in the absence (**A**) or presence (**B**) of 10 pmol of a *lac* operator carrying competitor DNA. (**C**) The topoisomers were incubated with 200 ng of *lac* repressor in the presence of 3 mM IPTG for 3 h. Incubation, electrophoresis and autoradiography were performed as described in Materials and methods. Positions of the free nicked minicircles (nic) and the free +1 topoisomers (+1) are indicated.

Loop formation between differently spaced operators within DNA minicircles of ΔLk = −2

The same type of analysis was performed with the −2 topoisomers (Figure 5). We found that all the complexes were extremely stable. Even after 11 h of dissociation the amount of free DNA was very small (Figure 5B). 200 ng of *lac* repressor were necessary to obtain the demonstrated degree of complex formation with the relaxed and the −1 topoisomers in the presence of 3 mM IPTG (Figures 3C and 4C). In contrast, 40 ng of *lac* repressor were sufficient to bind >60% of each of the −2 topoisomers in the presence of 3 mM IPTG (Figure 5C). The percentage of complex formation was even higher at the maximum of stability, somewhere in the range of operator distances of 161−165 bp. To ensure that the complexes had not formed in the gel after DNA and *lac* repressor were separated from IPTG, we performed the same experiment including 1 mM IPTG in the electrophoresis buffer. We could not find a significant difference in the results (data not shown).

The increased stability of the complexes between *lac* repressor and the −2 topoisomers over those formed with relaxed and −1 topoisomers is evident from dissociation experiments and the complex formation in the presence of IPTG. We are not able, however, to exactly quantify this increase from our results due to the extremely high affinities which result from the use of two 'ideal' *lac* operators.

Loop formation between differently spaced operators within DNA minicircles of ΔLk = +1

We also used positively supercoiled +1 topoisomers to look at DNA loop formation. The electrophoretic mobilities of these complexes are compared in Figure 6A. As the complexes are less stable than those obtained with the −1 topoisomers, their differential stability was even more clear. Only diffuse bands can be observed at operator distances around

Table I. Comparison between calculated and observed helical repeat

Topoisomer	ΔLk = +1	ΔLk = 0	ΔLk = −1	ΔLk = −2
Linking number[a]	44	43	42	41
Theoretical helical repeat[b]	10.27	10.51	10.76	11.02
Optimal spacing for loop stability[c]	154;165	157/158	161/162	163
Empirical helical repeat[d]	10.29 ± 0.03	10.50 ± 0.03	10.76 ± 0.03	10.87 ± 0.06
Least favourable spacing for loop stability[e]	160	163	156/157	158
Empirical helical repeat[f]	10.32 ± 0.03	10.52 ± 0.03	10.79 ± 0.03	10.89 ± 0.06

[a]To assign the linking number we assumed that the relaxed minicircles have a linking number of 43. This is in agreement with the helical repeat of 10.56 ± 0.03 bp found for relaxed DNA under 'normalized' conditions (25°C; ionic strength 60 mM) (Shore and Baldwin, 1983b; Horowitz and Wang, 1984; Goulet *et al.*, 1987).

[b]The helical repeat *h* was calculated from the equations Lk = Tw + Wr (2) with the assumption that Wr is negligible (see Discussion) and Tw = *N*/*h* (3) where *N* is the number of base pairs of the minicircle.

[c]The optimal spacings (in bp) between the two *lac* operators for loop stability were determined from at least two experiments as shown in Figures 3−6.

[d]The helical repeat was calculated from the observed maxima of loop stability for the different topoisomers assuming that they correspond to situations where the two *lac* operators have a distance of integral numbers of helical repeats. The margin of error results from the fact, that the maxima and minima can not be defined more precisely than ± 0.5 bp and even ± 1 b for the −2 topoisomers.

[e]The least favourable spacings (in bp) between the two *lac* operators for loop stability were determined from at least two experiments as shown in Figures 3−6.

[f]The helical repeat was calculated from the observed minima of loop stability. For details see d.

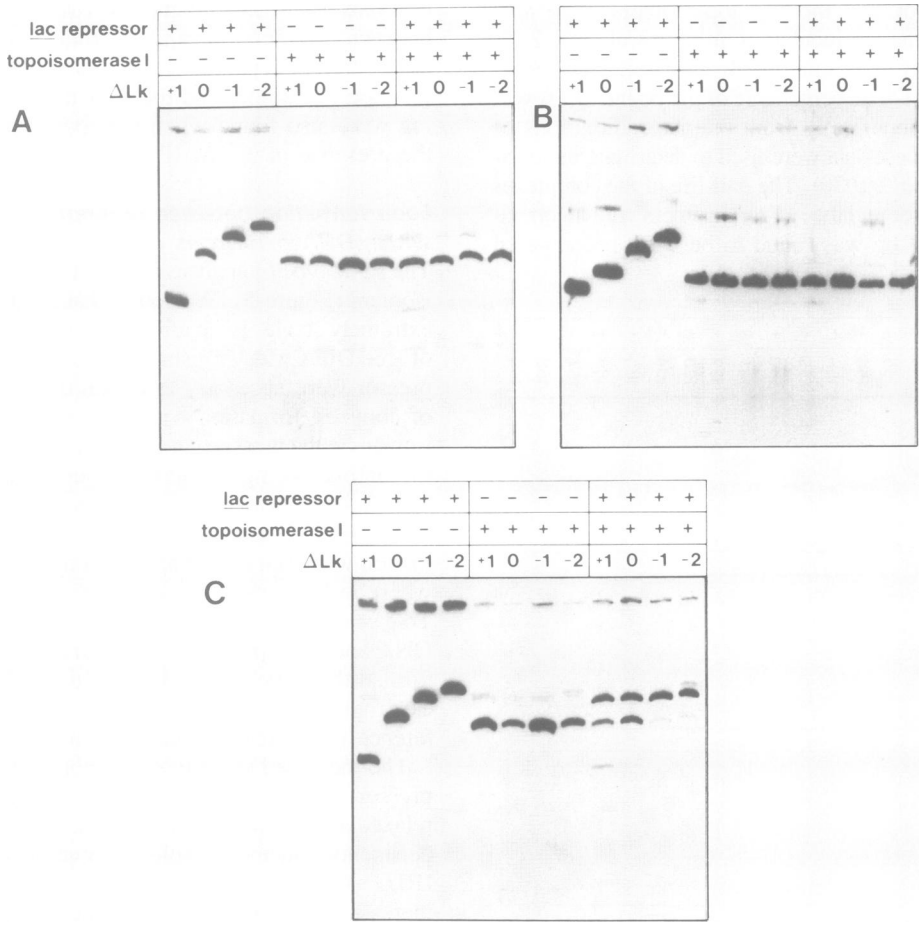

Fig. 7. *lac* repressor induces topological alterations within DNA minicircles. Purified topoisomers (~0.5 ng) with the indicated linking number difference (Lk) were incubated in the absence (−) or presence (+) or 40 ng of *lac* repressor for 15 min. Ten units of topoisomerase I were added where indicated and incubation was continued for 3 h. The deproteinized DNA minicircles were analysed on a 4% polyacrylamide gel containing 0.3 μg/ml ethidium bromide to allow separation of relaxed and nicked DNA minicircles (cf. Materials and methods). The DNA minicircles carried one 'ideal' *lac* operator (**A**) or two 'ideal' *lac* operators at a distance of 158 bp (**B**) and 163 bp (**C**).

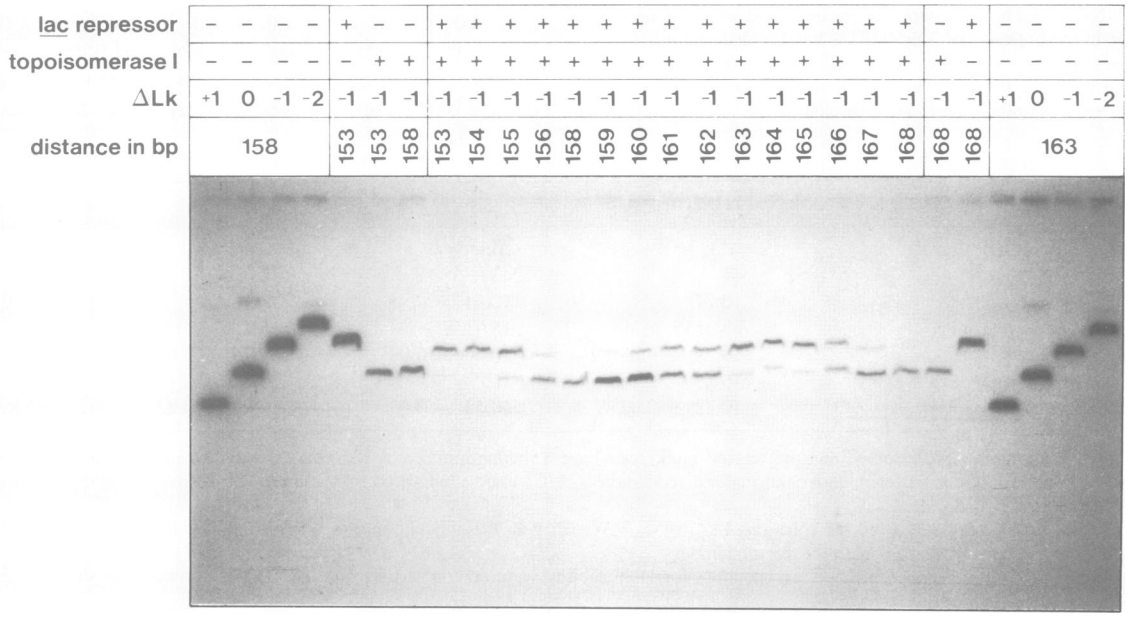

Fig. 8. The generation of topological alterations by *lac* repressor depends on the distance between the two *lac* operators. Purified −1 topoisomers (~0.3 ng) were incubated in the presence (+) or absence (−) of 20 ng of *lac* repressor for 15 min. Ten units of topoisomerase I were added where indicated and incubation was continued for 3 h. The deproteinized DNA minicircles were analysed on a 4% polyacrylamide gel containing 0.3 μg/ml ethidium bromide to allow separation of nicked and relaxed DNA minicircles (cf. Materials and methods). The +1, 0, −1 and −2 topoisomers with distances of 158 and 163 bp between the two *lac* operators were used as markers.

160 bp. This corresponds to the new minimum in stability of the complexes (Figure 6B and C). Correspondingly, the maxima were now centred at 154 and 165 bp. Thus the shift in spacing required for stable DNA loop formation is opposite for negative and positive supercoiling (Table I).

DNA looping affects topology of DNA

In order to investigate whether DNA looping affects the topology of the DNA, we performed topoisomerase I relaxation assays. We first looked at the binding of *lac* repressor to one 'ideal' *lac* operator. If this unwinds DNA significantly the distribution of the topoisomers after relaxation by topoisomerase I in the presence and absence of *lac* repressor should be different. Figure 7A demonstrates that this is not the case. Similarly, minicircles carrying two operators at a distance of 158 bp, which is an optimal distance for DNA looping in relaxed DNA (Figure 3), did not reveal any difference after relaxation of free and bound DNA when subjected to the same assay (Figure 7B).

In contrast, relaxation of complexes between minicircles with an operator spacing of 163 bp *lac* repressor and mainly yielded the -1 topoisomer, no matter with which topoisomer the incubation was started (Figure 7C). This indicates that under these conditions about one superhelical turn can be constrained by DNA looping. Figure 8 shows how this ability of DNA loops depends on the spacing between the two *lac* operators. It is evident that identical relaxation products in the presence and absence of *lac* repressor were obtained only in those cases where the two *lac* operators are best spaced for DNA looping with reference to relaxed DNA (158 and 168 bp). When the operator-spacing deviates from these values mixtures of relaxed and -1 topoisomers were obtained after the relaxation of the complexes. The percentage of the -1 topoisomers is roughly proportional to this deviation with maxima at distances of 153 and 163 bp between the two *lac* operators (Figure 8). These data suggest that *lac* repressor separates the DNA minicircle into two topological domains that cannot always be relaxed separately.

Discussion

It is well known that negative supercoiling increases the affinity of *lac* repressor for *lac* operator (Wang *et al.*, 1974; Wang *et al.*, 1982). This was believed to result from a small unwinding (0.15 turns) of natural *lac* operator due to the binding of *lac* repressor (see also Kim and Kim, 1982). We could not detect such unwinding in our experiments, but we do not know the lower limit of detection for unwinding in our assay (Figure 7A). We used 'ideal' *lac* operator, which is missing the central base pair (Sadler *et al.*, 1983; Simons *et al.*, 1984). Thus the known points of contact between *lac* repressor and operator (Lehming *et al.*, 1987) are even closer together, suggesting that in this case even greater unwinding might occur. The initial study was done with wild-type *lac* DNA carrying two additional pseudo-operators, which are now known to be able to participate in DNA looping between *lac* repressor and supercoiled *lac* DNA (Borowiec *et al.*, 1987; Whitson *et al.*, 1987a,b). Such a DNA loop may also account for the small supercoiling-dependent increase in affinity of *lac* repressor to the topoisomers carrying only one 'ideal' *lac* operator, as the third operator is also present in our minicircles (Figure 1A and Results). Since this weak binding site is not occupied when loop formation can occur between stronger binding sites (Borowiec *et al.*, 1987; Nick and Gilbert, 1985) the presence of the third operator in our constructs does not disturb our analysis.

How does DNA supercoiling influence DNA looping?

We observe two ways in which DNA supercoiling influences DNA loop formation. First, DNA supercoiling determines the distance between operators optimal for loop formation (i). Second, negative, but not positive DNA supercoiling, significantly increases the stability of DNA loops (ii).

(i) We assume that the optimal distance between two operators, defined by the maximum in stability of the complexes, is in part a reflection of the helical orientation of the two binding sites with respect to one another. Two binding sites positioned on the same face of the double-helix, i.e. separated by an integral number of helical turns, will most easily permit loop formation (Dunn *et al.*, 1984; Krämer *et al.*, 1987; this work). Our observation that DNA supercoiling is associated with a shift in distance optimal for DNA loop formation between two operators suggests to us that the helical repeat is altered by DNA supercoiling. As shown in Table I, we calculate from our binding data the helical repeats of 10.30, 10.51, 10.77 and 10.88 to be associated with minicircles of ΔLk values of $+1$, 0, -1 and -2 respectively. From the equations $Lk = Tw + Wr$ (2) and $Tw = N/h$ (3), where N is the size of the minicircles in bp, it follows that the helical repeat h is dependent on the partitioning of the linking difference into twist and writhe. Our experimentally derived values of the helical repeats are in very close agreement with the calculated values that one obtains upon assuming that writhe, Wr, is negligible in small DNA circles (Table I). Our data would therefore argue that for small DNA circles an existing linking number difference is largely causing changes in twist without simultaneously affecting writhe. Alternatively, *lac* repressor stabilizes such a conformation if it is in equilibrium with a writhed form. This interpretation is in agreement with the theory of Le Bret (1979, 1984), which predicts that DNA minicircles can adopt a totally untwisted conformation provided that the linking number difference does not exceed ~ 2 units. Otherwise, writhed conformations are preferred. Note that it was possible to observe the -1 topoisomers on electron microscopy grids as an entire population of open circles, like the relaxed topoisomers in the binding buffer (data not shown). The distribution of topoisomers obtained upon ligation of small DNA fragments (Shore and Baldwin, 1983a; Horowitz and Wang, 1984) can also be explained best by assuming the exclusive contribution of thermal fluctuations in twist. This is in line with Le Bret's theory as pointed out by Shore and Baldwin (1983b). For large DNA circles (>2000 bp) different values for the ratio $\Delta Wr/\Delta Tw$ have been obtained by different methods (Vologodskii *et al.*, 1979; Horowitz and Wang, 1984; Brady *et al.*, 1987). The same assay we used for DNA minicircles should in principle allow the determination of the partitioning of writhe and twist in larger DNA circles. Such experiments are in progress.

(ii) The stability of the DNA loops increased in going from the $+1$ and relaxed topoisomers to the -2 topoisomers. In other words, loop formation is not encouraged by positive supercoiling, whereas it is enhanced by increasing negative supercoiling. We do not believe that this is exclusively due to the intrinsic bending provided by negative supercoiling. For example, the half-life of the most stable complexes be-

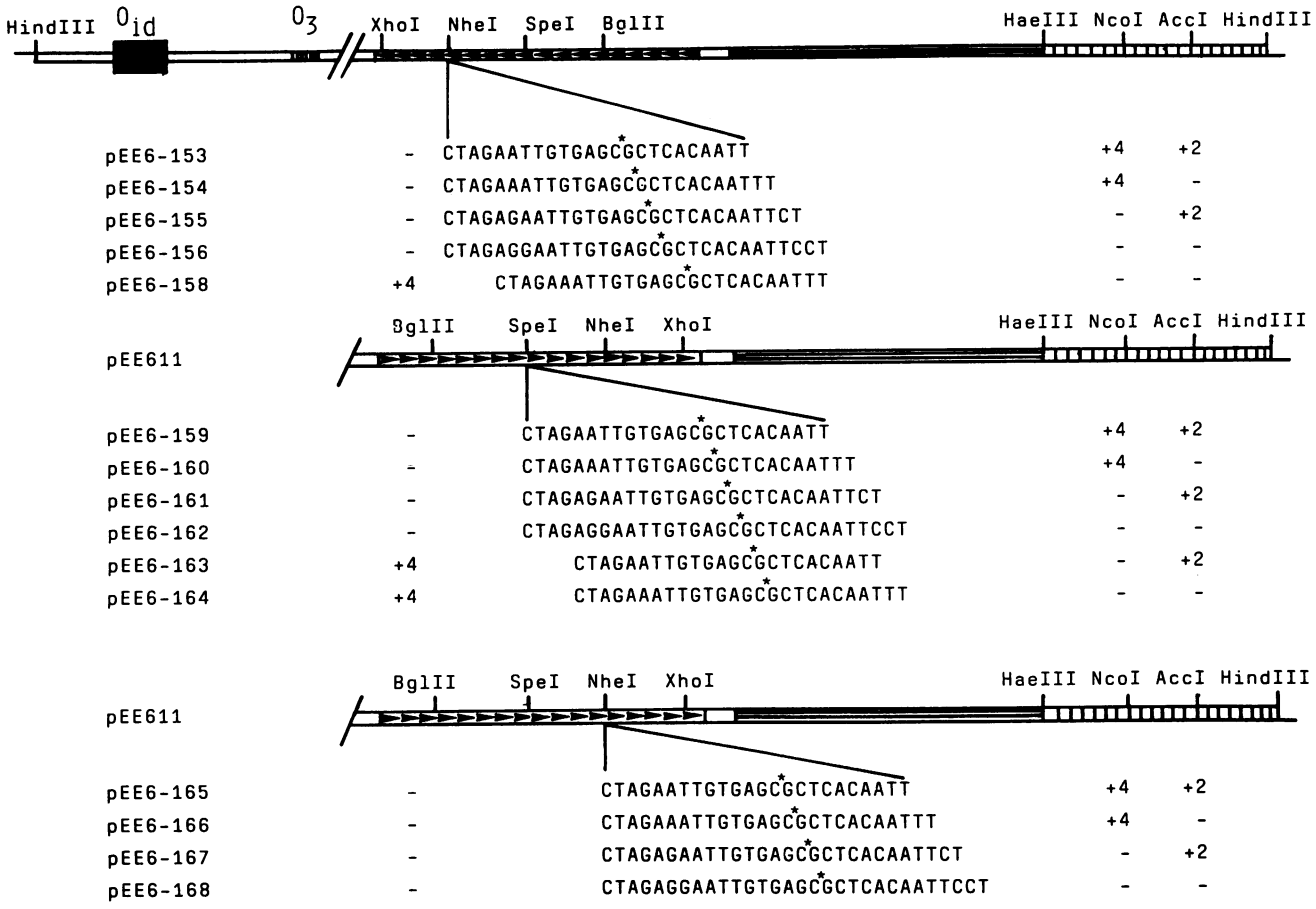

Fig. 9. Schematic representation of the DNA fragments used for the circularization. The open bar ▭ represents a 190-bp fragment derived from the *lac* promoter region. The wild-type first operator is replaced by an ideal *lac* operator (O_{id}), which is 94 bp apart from the third *lac* operator (O_3) at the 3′ end of the *lac* I gene (Besse *et al.*, 1986). Self-complementary oligonucleotides of 24, 26, 28 and 30 bp carrying ideal *lac* operators were inserted either into the *Nhe*I site of pK0610 or into the *Spe*I or *Nhe*I sites of pK0611, which differ only in the orientation of the polylinker ◀◀ inserted into the *Pvu*II site at the 3′ end of the *lac* I gene (Besse *et al.*, 1986; Krämer *et al.*, 1987). The *Xho*I site or the *Bgl*II site were additionally filled in (+4) to obtain the distances of 158 bp, or 163 and 164 bp respectively. These fragments together with a 140-bp fragment from pBR322 ▬ were inserted into the polylinker of pEE4 ▥ (Eismann *et al.*, 1987). Where indicated, the *Nco*I site (+4) or the *Acc*I site (+2) or both of them were filled in, in order to compensate for the different length of the fragments. The 452-bp fragments which were excised with *Hin*dIII and used for circularization are not drawn to scale.

tween *lac* repressor and the −1 topoisomers (161 bp) is at least 10 times higher than that of the most stable complexes with relaxed DNA (158 bp), although no significant additional writhing is expected to occur (see above).

Local alterations in DNA structure can occur within DNA loops, as evidenced by alterations in the sensitivity to various chemical or enzymatic probes of DNA structure in footprinting experiments (Hochschild and Ptashne, 1986; Borowiec *et al.*, 1987; Krämer *et al.*, 1987). Negative supercoiling may provide the energy to promote these structural alterations ultimately leading to DNA bending and loop stabilization (Borowiec *et al.*, 1987). Positive supercoiling would not encourage such DNA polymorphism. Another possibility is that untwisting of the DNA helix increases its flexibility. In contrast, more tightly wound DNA, as would result from positive supercoiling, would intuitively appear to be more rigid.

Nitrocellulose filter binding has been recently used to deduce a strong increase of stability of DNA loops between *lac* repressor and two *lac* operators by DNA supercoiling (Whitson *et al.*, 1987a,b; Hsieh *et al.*, 1987). Part of the interpretation of these data seems questionable since it has been demonstrated (Besse *et al.*, 1986) that such complexes with linear DNA bind very poorly to nitrocellulose filters. For example, the small (4-fold) increase of half-life for a complex between *lac* repressor and linear DNA due to an additional *lac* operator at a distance of 305 bp (29.0 helical turns) (Hsieh *et al.*, 1987) is more in line with the formation of tandem structures than with the formation of DNA loops, which show at least a 1000-fold increase of half-life at a distance of 221 bp (21.0 helical turns) between the two operators (Besse *et al.*, 1986).

DNA supercoiling diminishes the spacing requirement for DNA looping

Increasing negative supercoiling eases the requirement for correct spacing between two operators in our system. Figure 5 demonstrates that only small differences in stability exist between the most stable and unstable loops formed in −2 topoisomers of different operator spacing. We believe that this is in part due to a variation of the ratio $\Delta Wr/\Delta Tw$ in the different −2 topoisomers, which results in a mixed population of molecules. In line with this reasoning, a variety of forms from the open circle to a several-fold interwound structure could be observed from an individual −2 topo-

isomer in electron microscopy (data not shown). *lac* repressor, upon loop formation, may stabilize that conformation, in which the two operators are separated by a nearly integral number of helical turns. We do not know what would be the magnitude of this effect on larger DNA circles.

DNA looping affects topology of the DNA

The formation of high order protein−DNA complexes is not only relevant to the regulation of transcription but also for replication and site-specific recombination (Gellert and Nash, 1986; Echols, 1986; Richet *et al.*, 1986). Echols proposed that these specialized nucleoprotein complexes might produce structural changes in the DNA duplex which are essential for their biological function. We demonstrate that one superhelical turn can be found constrained upon loop formation by *lac* repressor. This is not a consequence of loop formation *per se*, but depends strongly on the distance between two operators and therefore on their geometrical positioning towards each other (Figure 8). Thus, the simultaneous binding of *lac* repressor to two *lac* operators can create local DNA domains with altered topological properties. That the formation of such topological domains by *lac* repressor influences the recombinational activity of *res* sites within this domain has been demonstrated (Saldanha *et al.*, 1987).

Does DNA supercoiling influence DNA looping in vivo?

DNA in *E.coli* is known to contain unrestrained superhelicity (Pettijohn and Pfenninger, 1980). The superhelicity was estimated by different methods to be in the range of −0.025 (Greaves *et al.*, 1985; Peck and Wang, 1985) to −0.046 (Borowiec and Gralla, 1987). DNA supercoiling *in vivo* may help to bring two protein binding sites into the correct spacing for DNA looping. This might be especially relevant when additional parameters, like ionic strength or binding of histone like proteins, influence the helical twist. Furthermore DNA supercoiling can generally stabilize DNA loops and thus allow more efficient repression. For instance, both pseudo-operators in the *lac* operon have been reported to participate in loop formation with the first operator in supercoiled DNA (Whitson *et al.*, 1987a,b; Borowiec *et al.*, 1987). Our results provide an explanation for the low affinities of the two *lac* pseudo-operators in that full inducibility of the *lac* operon must be ensured under the conditions of *in vivo* DNA supercoiling. Therefore, subtle changes in local superhelical stress can be envisaged to contribute to mechanisms of repression as well as derepression of the *lac* operon *in vivo*.

The interplay between DNA looping and supercoiling may also be relevant in eukaryotic cells where it has been proposed that chromatin is organized in topologically constrained, looped domains, the superhelicity of which differs between active and inactive domains (reviewed in Gasser and Laemmli, 1987; Gross and Garrard, 1987). Multiprotein DNA complexes at promoters and upstream regulatory regions are involved in the regulation of transcription in eukaryotic cells (see for example Cereghini *et al.*, 1987; Maniatis *et al.*, 1987). It seems possible that superhelicity *in vivo* not only facilitates the formation of such nucleoprotein structures (Richet *et al.*, 1986) but also influences their spatial orientation towards each other by supercoil-induced changes in helical twist.

Materials and methods

Chemicals and enzymes

Chemicals are the same as listed in Krämer *et al.* (1987). Restriction endonucleases and other enzymes were obtained from Boehringer (Mannheim, FRG), New England Biolabs (Beverly, USA), Bethesda Research Laboratories (Neu-Isenburg, FRG) and Pharmacia (Freiburg, FRG), and were used as recommended by the manufacturers. Purified *lac* repressor was a gift of K.Beyreuther. Netropsin was a gift of Dr Lunel, Rhone-Poulenc.

Construction of plasmids

The plasmids pEE611 and the series from pEE6-153 to pEE6-168 were obtained by inserting *Eco*RI−*Hae*III restriction fragments (~400 bp) containing the modified *lac* promoter regions of the plasmids pK0611 and the series from pK06153 to pK06168 (Krämer *et al.*, 1987) between the *Eco*RI and *Stu*I restriction sites of the plasmid pEE4 (Eismann *et al.*, 1987) or derivatives of this plasmid (Figure 9). In order to compensate for the differences in length of the various *lac* operator oligonucleotides used for the construction of the pK0-series the length of the pEE4 polylinker was altered by filling in either the *Acc*I site (2 bp) or the *Nco*I site (4 bp) or both (6 bp). After this modification a 452 bp *Hin*dIII−*Hin*dIII restriction fragment could be obtained from all these plasmids with the exception of pEE611 (422 bp). The constructions were obtained using standard procedures (Maniatis *et al.*, 1982) and were verified by restriction mapping or sequence analysis (Maxam and Gilbert, 1977; Chen and Seeburg, 1985). Distances between the two operators were counted from one centre of symmetry to the other.

Circularizations

The 452 bp *Hin*dIII−*Hin*dIII fragments (or 422 bp fragment from pEE611 respectively) were dephosphorylated with alkaline phosphatase, purified and labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labelled fragments (< 100 ng/ml) were ligated in 50 mM Tris−HCl (pH 8.0), 6 mM $MgCl_2$, 20 mM DTT and 1 mM ATP with 1 U T4 DNA ligase/ml for 12 h at room temperature (Nordheim and Meese, 1988). Ethidium bromide (0.5 or 1 μM) was added to the incubation buffer to obtain predominantly the −1 or the −2 topoisomers respectively (Wang, 1974). In order to obtain the +1 topoisomer ligation was performed in the presence of 10 μM Netropsin at 16°C (Malcolm and Snounou, 1982; Zivanovic *et al.*, 1986). After ligation the DNA was extracted with phenol, precipitated with ethanol and subjected to PAGE to purify the individual topoisomers (Zivanovic *et al.*, 1986; Nordheim and Meese, 1988).

The topoisomers were identified by their consecutive appearance in ligation reactions containing gradually increasing amounts of ethidium bromide (Nordheim and Meese, 1988). The topoisomer which was obtained in the ligation reaction without any DNA binding drug comigrated with the nicked minicircles, indicating that it was near to relaxation. This topoisomer (ΔLk = 0) was chosen as a reference.

Gel electrophoresis

Gel electrophoresis was performed as described by Krämer *et al.* (1987) with minor modifications. The respective minicircles (~0.1 ng) were incubated in 10 μl binding buffer (BB) with the indicated amounts of *lac* repressor in the presence or absence of 3 mM IPTG. Binding buffer was 10 mM Tris−HCl (pH 8.0), 10 mM KCl, 10 mM MgAc, 0.1 mM EDTA, 0.1 mM DTT, 50 μg/ml bovine serum albumin and 5 μg/ml sonicated salmon sperm DNA (Riggs *et al.*, 1970). After 15 min at room temperature, 3 μl of 15% ficoll in BB with 0.06% bromophenol blue and 0.06% xylene cyanol were added. When IPTG was present the incubation was done for 3 h to ensure that equilibrium was reached.

Four percent polyacrylamide gels (acrylamide to bisacrylamide was 30:1) in 45 mM Tris/borate, 1.5 mM EDTA, pH 8.3 were prerun for at least 2 h at a voltage gradient of 12 V/cm at room temperature. When all the samples had been unloaded, electrophoresis was performed for 5−6 h under the same conditions. For the separation of the −1 topoisomers and their complexes with *lac* repressor the gel system was modified by the addition of 5 mM $MgCl_2$. In this case the voltage gradient was 9 V/cm. In order to differentiate between the nicked and the relaxed topoisomers after the topoisomer I relaxation assays, the electrophoresis was performed in the presence of 0.3 μg/ml ethidium bromide.

To measure dissociation rates of preformed complexes between *lac* repressor and the respective minicircles 1 μl of BB containing 10 pmol of competitor DNA carrying one 'ideal' *lac* operator was added to the incubation mixture. Competitor DNA was derived from the 36 bp self-complementary oligonucleotide 5′ GAATTCAATTGTGAGCGCTCACAATTGAATTC-TGCA 3′. The underlined bases correspond to the 'ideal' *lac* operator. The oligonucleotide was synthesized on an Applied Biosystems 380 A DNA

synthesizer and purified on a denaturing polyacrylamide gel. After the indicated time dissociation was stopped by applying the incubation mixture to a running gel. After electrophoresis, gels were dried and autoradiographed at −70°C on Kodak X-Omat AR film.

For quantitative determinations the autoradiographs were scanned on a Bio-Rad video densitometer. The percentage of bound DNA was calculated for the various time points and dissociation rates were determined according to Riggs *et al.* (1970).

Topoisomerase I relaxation experiments

Topoisomerase I relaxation experiments were carried out as described (Amouyal and Buc, 1987). In detail, ~0.5 ng of the respective minicircles were incubated in the presence or absence of 40 ng *lac* repressor in 10 μl BB at room temperature. Topoisomer gel retardation assays were used to ensure that >95% of the DNA was bound under these conditions. Ten units of topoisomerase I (BRL) were added and incubation was continued for 3 h at room temperature. The mixture was deproteinized with phenol, precipitated with ethanol, dried and subjected to gel electrophoresis in the presence of 0.3 μg/ml ethidium bromide.

Electron microscopy

Electron microscopy was performed as described by Krämer *et al.* (1987).

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